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Distribution of the Rad51 recombinase in human and mouse spermatocytes

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***In vitro*, the human Rad51 protein (hRad51) promotes homologous pairing and strand exchange reactions suggestive of a key role in genetic recombination. To analyse its role in this process, polyclonal antibodies raised against hRad51 were used to study the distribution of Rad51 in human and mouse spermatocytes during meiosis I. In human spermatocytes, hRad51 was found to form discrete nuclear foci from early zygotene to late pachytene. The foci always co-localized with lateral element proteins, components of the synaptonemal complex (SC). During zygotene, the largest foci were present in regions undergoing synapsis, suggesting that Rad51 is a component of early recombination nodules. Pachytene nuclei showed a greatly reduced level of Rad51 labelling, with the exceptions of any asynapsed autosomes and XY segments, which were intensely labelled. The distribution of Rad51 in mouse spermatocytes was similar to that found in human spermatocytes, except that in this case Rad51 was detectable at leptotene. From these results, we conclude that the Rad51 protein has a role in the interhomologue interactions that occur during meiotic recombination. These interactions are spatially and temporally associated with synapsis during meiotic prophase I.**

Keywords: human/meiosis/recombination/recombination nodule/synaptonemal complex

Introduction

Meiosis is the process by which haploid gametes are produced from diploid parental cells. The subsequent union of gametes to form a zygote restores the normal diploid chromosome number. During the first meiotic division, replicated chromosomes move to opposite poles; in the second meiotic division sister chromatids move to opposite poles. In most organisms, reciprocal recombination also provides a physical link between homologous chromosomes, thus ensuring the proper distribution of chromosomes at meiosis I.

The cytological events occurring in meiotic prophase I have been well documented for many organisms (von Wettstein *et al*., 1984). During leptotene, each pair of sister chromatids develop a common proteinaceous core called an axial element. Later, in zygotene, the protein components of the central region of the synaptonemal complex (SC) begin to assemble between the axial elements of the two pairs of sister chromatids, which become the lateral elements of the incomplete synaptonemal complex. At this stage, abundant electron-dense structures termed zygotene nodules, or early recombination nodules, are observed to be associated with the lateral elements of SCs (Albini and Jones, 1987; Carpenter, 1988). The pachytene stage begins when synapsis of chromosomes is completed, resulting in the presence of full-length SCs. Nodules present during pachytene are termed late recombination nodules (Carpenter, 1988). Meiosis I proceeds through diplotene, in which homologues begin to repel and the chiasmata become visible, and further to diakinesis in which the chromosomes coil and contract, reaching their most condensed state by metaphase I. Homologues disjoin at anaphase I.

Whereas the cytological changes occurring during meiosis have been well studied in many organisms, the molecular events that take place in meiotic recombination have been studied almost exclusively in the budding yeast *Saccharomyces cerevisiae* (reviewed by Roeder, 1995). This is primarily because *S.cerevisiae* can be induced to undergo meiosis in a synchronous manner, allowing temporal analyses of cytological, biochemical and molecular events. Secondly *S.cerevisiae* is amenable to genetic modifications that facilitate the analysis of mutants, and thirdly the four products of individual meioses can be isolated and analysed.

In *S.cerevisiae*, recombination proceeds concurrently with assembly of the SC. Meiotic recombination is initiated in most, if not all, cases by the introduction of double-strand breaks (DSBs) at specific sites, that are at least partially influenced by chromatin structure (Ohta *et al*., 1994; Wu and Lichten, 1994). DSBs are subjected to 5′–3′ exonucleolytic processing, resulting in molecules with 3′ single-stranded tails that can be detected in leptotene before formation of the SC, and which disappear in early pachytene as they are converted to intermediates containing double Holliday junctions (Padmore and Kleckner, 1991; Schwacha and Kleckner, 1995). Double Holliday junction intermediates can be detected throughout most of pachytene and then disappear with the coincident appearance of mature crossover and non-crossover recombination products (Goyon and Lichten 1993; Schwacha and Kleckner, 1995). Crossover products are resolved as chiasmata after disassembly of the SC (Carpenter, 1994).

Characterization of *S.cerevisiae* mutants has led to the identification of several genes involved in meiotic recombination. These include those of the *RAD52* epistasis group and the *XRS2* and *MRE11* genes, originally characterized as DNA repair defective, and *DMC1* which exhibits

Table I. Numbers of hRad51 foci during zygotene and pachytene

	Early zygotene	Zygotene	Pachytene (including XY foci)	Pachytene (excluding XY foci)	Late pachytene (including XY foci)	Late pachytene (excluding XY foci)
n	20	28	49	49	9	9
Mean (SD)	110.2 (23)	152.1 (45.1)	47.8 (21.7)	30.4 (20.9)	10.4 (14.7)	5.3 (8.4)
Range	75–150	91–262	18–119	8–87	0–37	0–20

Cells in which only the terminal regions of chromosomes were visible were classed as early zygotene cells. Cells in which at least one bivalent was visible from terminus to terminus (including interstitial regions of asynapsis) were considered zygotene. Cells in which all autosomes were synapsed and the axis of the X and Y remained clear were classed as pachytene, and cells in which the axis of the X and Y had become split and anastamosed were classed as late pachytene.
n = number of cells analysed; SD = standard deviation.

a meiosis-specific recombination defect (Game, 1983; Bishop *et al*., 1992; Ivanov *et al*., 1992; Ajimura *et al*., 1993). Whilst the study of the biochemical reactions promoted by their protein products is very much in its infancy, the analysis of DNA intermediates that accumulate *in vivo* provides important clues to their biochemical roles. For example, *rad50, xrs2* and *mre11* mutants are defective in DSB formation and *rad51* and *dmc1* mutants accumulate intermediates with 3′ single-stranded termini (Alani *et al*., 1990; Bishop *et al*., 1992; Ivanov *et al*., 1992; Shinohara *et al*., 1992; Johzuka and Ogawa, 1995).

RAD51 and *DMC1* encode homologues of the *Escherichia coli* RecA protein (Shinohara *et al*., 1992; Bishop, 1994). The 38 kDa RecA protein forms nucleoprotein filaments on DNA and catalyses homologous pairing and strand exchange reactions *in vitro* (Eggleston and West, 1996). Similar *in vitro* reactions are promoted by the yeast (Sung, 1994; Sung and Robberson, 1995) and human (Benson *et al*., 1994; Baumann *et al*., 1996) Rad51 proteins. Homologues of RecA protein have also been identified in *Drosophila melanogaster* (McKee *et al*., 1996), *Lillium longiflorum* (Terasawa *et al*., 1995), *Xenopus laevis* (Maeshima *et al*., 1995), chicken (Bezzubova *et al*., 1993) and mouse (Morita *et al*., 1993; Shinohara *et al*., 1993). Expression of the *RAD51* gene is elevated in meiotic tissue of mouse and chicken, suggestive of a role in meiotic recombination (Bezzubova *et al*., 1993; Morita *et al*., 1993). Interestingly, the *RAD51* gene is expressed at high levels in *D.melanogaster* only in female meiotic tissue (McKee *et al*., 1996). In male meiotic tissues, in which recombination is completely absent, *RAD51* transcript levels are suppressed relative to bulk RNA (McKee *et al*., 1996). The *RAD51* gene was shown recently to be essential in the mouse since homozygous *RAD51*$^{-/-}$ knockouts exhibit an embryonic lethal phenotype (Tsuzuki *et al*., 1996).

Analysis of the distribution of Rad51 protein in *S.cerevisiae* meiotic cells indicates that it is a component of early recombination nodules, the proposed sites for initiation of strand invasion and chromosome synapsis (Bishop, 1994). Immunoelectron microscopic studies of lily meiocytes have localized RecA homologues to early recombination nodules (Anderson *et al*., 1997). Following immunofluorescence studies in mouse meiocytes, Plug *et al.* (1996) reported that Rad51 foci appear prior to lateral element proteins and that Rad51-associated sequences are the sites of initial contact between homologues. Plug *et al.* (1996) speculated that Rad51 acts (i) prior to synapsis in a homology search, a stage that occurs before the formation of DSBs, (ii) during synapsis and (iii) in crossing-over/reciprocal recombination. Further immunofluorescence studies of mouse spermatocytes have localized Rad51 foci to chromatin loops in leptotene and zygotene nuclei, with foci associated with SCs in pachytene nuclei (Ikeya *et al.*, 1996). In meiotic and mitotic cells, Rad51 appears to co-localize with Brca1, mutations in which are associated with breast cancer (Scully *et al*., 1997).

Here, we present the results of an immunocytogenetic study of Rad51 distribution in human spermatocytes and, to allow comparison with earlier data, mouse spermatocytes. We find that Rad51 localizes to regions of DNA that are undergoing, or are about to undergo, synapsis. The distribution of hRad51 in spermatocytes is remarkably consistent with the distribution of Rad51 in yeast, and indicates that Rad51 functions in meiotic recombination as a component of early recombination nodules. We find no evidence to support the proposals that Rad51 participates in a homology search occurring prior to synapsis or in late recombination nodules.

Results

Distribution of hRad51 in human spermatocytes in meiosis I

A sequential immunocytogenetic technique was used to study: (i) the distribution of hRad51 and centromeric proteins in human spermatocytes, and (ii) the distribution of lateral element (LE) proteins of the SC. The labelled centromeric proteins, which remain constant in both images, allow the images to be superimposed and provide reference points to allow the distribution of labelled Rad51 to be determined relative to LE proteins.

Zygotene. In normal human males, the leptotene stage, in which complete axial elements are visible prior to synapsis, is absent (Speed and Chandley, 1990). The earliest stage at which we have localized hRad51 is zygotene. Early zygotene nuclei were characterized by the presence of high numbers of labelled hRad51 foci (Table I). The hRad51 foci are visualized in white in Figure 1A-1, with the centromeres shown in red. When the same nuclei were visualized after labelling with antibodies specific for LE protein (Figure 1A-2), we observed that the hRad51 foci always co-localized with the newly polymerizing axial/lateral element material. The largest and brightest foci were visible either at the point of synapsis of two LEs (red arrow, Figure 1A), or at the termini of axial elements that were in close proximity and apparently about to

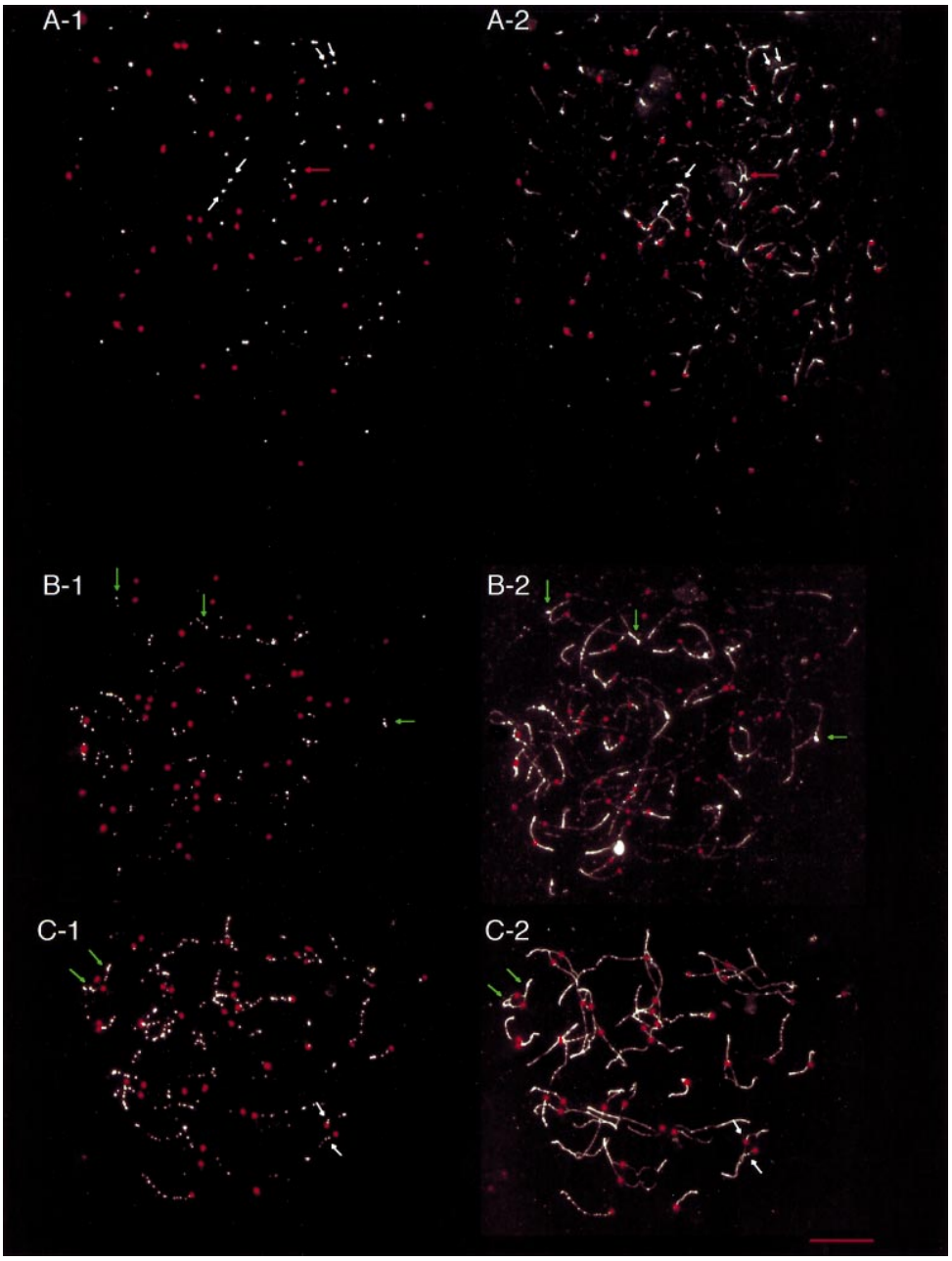


Fig. 1. Localization of hRad51 in human spermatocytes in zygotene. (**A**) Early zygotene spermatocyte from normal human Male 2 labelled in (A-1) with anti-hRad51 (white) and sera GS (centromeres, red) and in (A-2) with antibody A1 labelling (anti-lateral element; white) superimposed. Red centromere signals and arrows, which remain constant between the two images, can be used for orientation. White arrows indicate hRad51 foci at the terminus of axial elements that are apparently about to synapse; the red arrow indicates one hRad51 focus at the point of synapsis of two lateral elements. (**B**) and (**C**) Zygotene spermatocytes from normal Male 1 labelled as described for (A). Green arrows indicate large bright foci localized to synapsed regions, and white arrows indicate smaller less bright foci localized to asynapsed regions. Scale bar, 10 μm.

synapse (white arrows, Figure 1A). Smaller foci were visible on asynapsed axial elements. The presence of the larger foci of hRad51 in regions of synapsis strongly suggests that the role of the protein is purely 'short range' and that it is unlikely to be involved in the preceding 'long-range' pairing of homologous chromosomes.

Analysis of hRad51 in mid-zygotene cells revealed the presence of a greater number of foci (Table I), and these were found to be arrayed in obvious linear tracks (Figure 1B-1 and C-1). The linear arrays of foci co-localized with the axial/lateral element material, with the larger and brighter foci usually, but not exclusively, found in synapsed regions (green arrows, Figure 1B and C). Smaller, less bright, foci were localized to asynapsed regions (white arrows, Figure 1C).

At late zygotene, four or five large arrays of foci were visible, representing newly fully synapsed or synapsing bivalents (Figure 2A). Many bivalents at this stage were found to be unlabelled or were very lightly labelled, indicating that hRad51 labelling diminishes rapidly following synapsis. A single autosomal bivalent can be seen which is in the late stages of synapsis (Figure 2A, white arrow). This bivalent is heavily labelled in the synapsed regions whilst the small interstitial asynapsed region

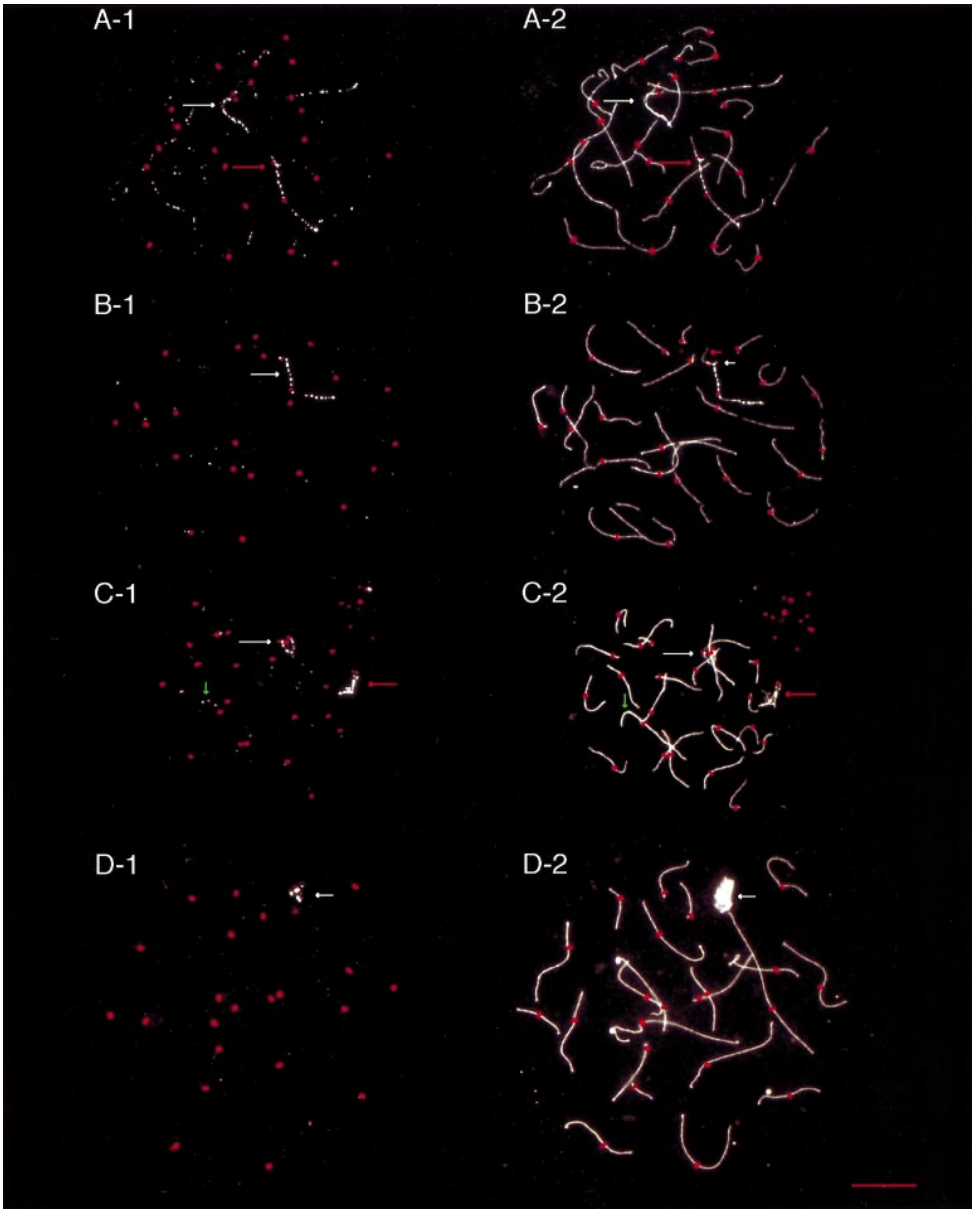


Fig. 2. Localization of hRad51 in human spermatocytes in late zygotene and pachytene. (A-1, B-1, C-1 and D-1) Spermatocytes from normal human Male 1 labelled with anti-hRad51 (white) and sera GS (centromeres, red). (A-2, B-2, C-2 and D-2) The same with labelling from antibody A1 (anti-lateral element; white) superimposed. The red centromere signals and the arrows, which remain constant between the two images, can be used for orientation. (**A**) Late zygotene cell. The white arrow indicates a single bivalent which is in the late stages of synapsis. The red arrow indicates the XY bivalent. (**B**) Early pachytene cell. The white arrow indicates a heavily labelled XY bivalent. The red arrow in (B-2) indicates the unlabelled region of the Y chromosome. (**C**) Pachytene cell. The red arrow indicates the XY bivalent. The white arrow indicates hRad51 labelling at a region of interstitial asynapsis of an autosomal bivalent. The green arrow indicates weakly labelled autosomal foci of anti-hRad51. (D) Late pachytene cell. The white arrow indicates the anastamosed and shredded XY bivalent. Scale bar, 10 μm.

remains unlabelled. At this stage, the asynapsed segments of the XY bivalent start to accumulate hRad51 (Figure 2A, red arrow), although the Y heterochromatic region (which protrudes at the top of the XY bivalent in Figure 2A-2) remains unlabelled.

Pachytene. By pachytene, the labelling of autosomes was greatly diminished, such that a much reduced number of weakly labelled hRad51 foci remained (Table I and Figure 2B and C). In early pachytene, any remaining linear arrays of foci co-localized with the now fully synapsed SC material. Later, the pattern of autosomal foci was unpredictable, with some bivalents entirely free of hRad51 whereas others showed several foci in close proximity (e.g. Figure 2C, green arrow). At this stage, the XY bivalent became increasingly conspicuous relative to the fully synapsed autosomes since the accumulation of hRad51, which began at late zygotene, continued into pachytene (Figure 2B-1, white arrow).

Exceptional nuclei, in which autosomal bivalents remained asynapsed by the pachytene stage, showed a distinct change in labelling pattern when compared with zygotene nuclei. In this case, asynapsed autosomal regions were found to be heavily labelled (Figure 2C, white arrow) and similar in appearance to the XY bivalent (Figure 2C, red arrow) whereas the synapsed regions exhibited the

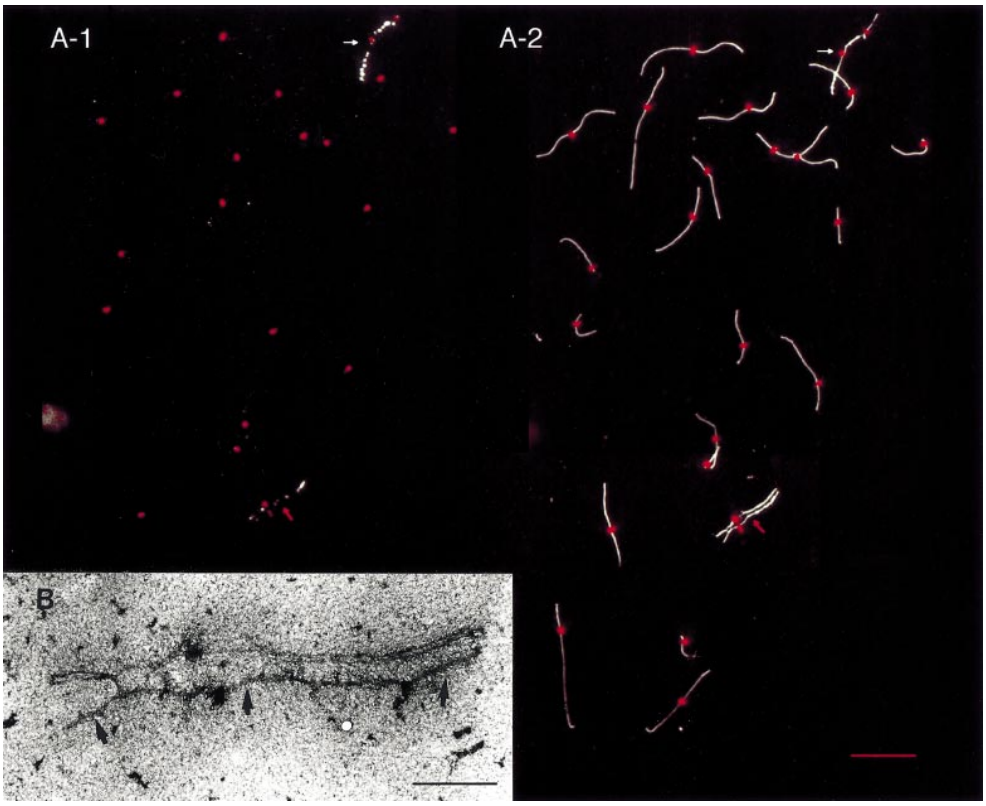


Fig. 3. Localization of hRad51 in human spermatocyte at pachytene. (**A**) Pachytene spermatocyte from normal human Male 2 labelled in (A-1) with anti-hRad51 (white) and sera GS (centromeres, red) and in (A-2) with antibody A1 labelling (anti-lateral element; white) superimposed. The red centromere signals and the arrows can be used for orientation. The white arrow indicates the XY bivalent. The red arrow indicates an apparent trivalent. Scale bar 10 μm. (**B**) The trivalent arrowed in (A) after PTA staining and transfer to TEM according to the technique of Messier *et al.* (1986). The black arrows indicate the thickened appearance of the supernumerary asynapsed axial elements. Scale bar, 2 μm.

usual reduction in the level of labelling. This pattern was confirmed in an apparently trisomic cell (Figure 3). Two regions of anti-hRad51 labelling were seen in this cell. One heavily labelled region corresponded to the XY bivalent (Figure 3A-1 and A-2, white arrow). The second region (Figure 3A-1 and A-2, red arrow) corresponded to an apparent trivalent, which was unable to complete synapsis, as shown by the anti-LE antibody. This interpretation was confirmed by transmission electron microscopy, following phosphotungstic acid (PTA) staining. Using this technique, we observed that the region of heavy anti-hRad51 labelling corresponds to a trivalent containing a largely asynapsed axial element (Figure 3B). This indicates that the heavy labelling seen on the asynapsed regions of the XY occurs purely as a consequence of asynapsis and is not a special feature of this bivalent.

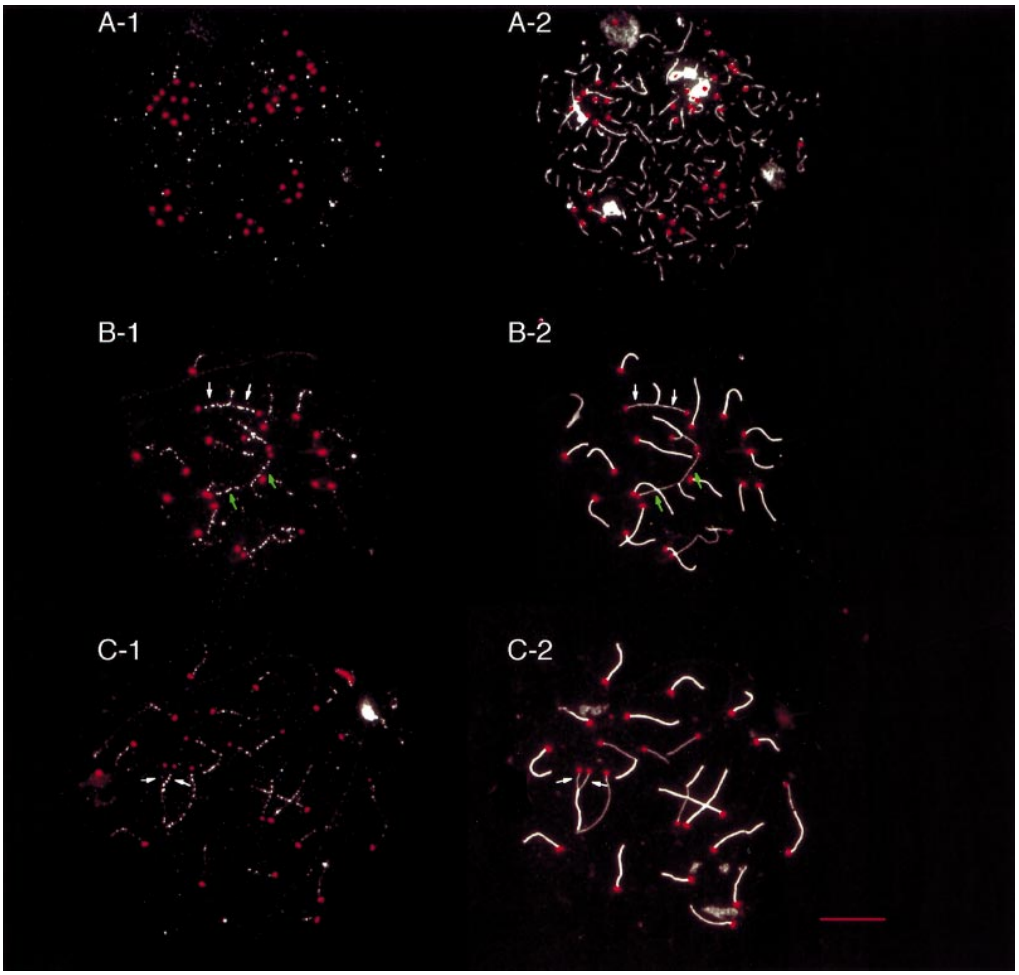
Late pachytene nuclei show a labelling pattern in which autosomal labelling has diminished still further (Table I and Figure 2D), although occasional foci may remain. The XY bivalent, which by this stage has become shredded and anastamosed (Solari, 1980), may remain distinctly labelled, although the number of the foci is reduced and their size increased (Figure 2D, white arrow).

Some late pachytene nuclei were detected in which no hRad51 labelling could be seen (data not shown). It is not clear whether such nuclei represent a very late stage, or failed to label for artefactual reasons. We tentatively conclude that by late pachytene few or no hRad51 foci remain. The absence of hRad51 from very late pachytene nuclei may explain why data could only be obtained from nine nuclei (Table I), because images were generally collected from nuclei in which hRad51 was detectable. Obviously, such unlabelled cells would remain inconspicuous at this stage.

Overall, the distribution of hRad51 in two normal human males was analysed using two independent preparations of anti-Rad51 polyclonal antibodies. The observed distribution of hRad51 was the same in each case, and was independent of the antibody used in the experiment. In addition, the distribution of hRad51 in spermatocytes from a third male was analysed using monoclonal antibodies (mAbs) raised against hRad51. Although higher levels of background labelling were observed with the mAb, the results corroborated those obtained with the polyclonal antibodies (data not shown).

Distribution of Rad51 in mouse spermatocytes

Since the human and mouse Rad51 proteins are 98.8% identical, we were able to use polyclonal antisera raised against hRad51 to study the distribution of Rad51 in mouse spermatocytes. In contrast to human males, male mice show considerable axial element formation prior to synapsis and have a more conspicuous leptotene stage (Guitart *et al.*, 1985). Such leptotene nuclei contain numerous foci of Rad51 which co-localized with asynapsed axial element material (Figure 4A). This represents the earliest stage at which we were able to detect Rad51 in male mice.



Fig, 4. Distribution of Rad51 in mouse spermatocytes. (A-1, B-1 and C-1) Zygotene spermatocytes from Balb C mouse labelled with anti-hRad51 (white) and sera GS (centromeres, red). (A-2, B-2 and C-2) the same with labelling from antibody A1 (anti-lateral element; white) superimposed. The red centromere signals and the arrows can be used for orientation. (**A**) Leptotene cell. (**B**) and (**C**) Zygotene cells. Arrows indicate Rad51 labelling of unsynapsed axial elements. Scale bar, 10 μm.

Zygotene nuclei contained numerous linear arrays of Rad51 foci. In contrast to the results obtained with human zygotene nuclei, heavy hRad51 labelling was found in both synapsed and asynapsed regions. The unsynapsed homologues did not appear to show the symmetry of labelling previously described (Ashley *et al*., 1995; Plug *et al*., 1996). To demonstrate this point, images were taken from three partially synapsed bivalents (Figure 5A-1, B-1 and C-1), and mirror images of each of the unsynapsed axial elements were created for comparison (Figure 5A-2, A-3, B-2, B-3, C-2 and C-3). The distribution of Rad51 on the unsynapsed regions of partially synapsed bivalents was clearly asymmetric.

In the mouse spermatocytes, the labelling of fully synapsed bivalents appeared to diminish very rapidly, with bivalents often lightly labelled or unlabelled in mid-to-late zygotene nuclei (Figure 4B and C). The pachytene nuclei showed a similar Rad51 distribution to that observed in humans, with the XY becoming conspicuously labelled and autosomes labelled only by a few faint foci (data not shown).

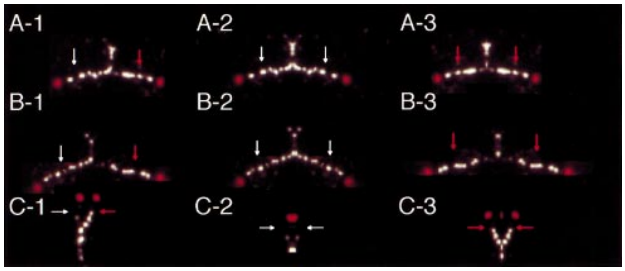


Figure 5. Asymmetry of Rad51 labelling. (**A-1**, **B-1** and **C-1**) Individual partially synapsed mouse bivalents labelled with anti-Rad51 (white) and serum GS (centromeres, red) derived from Figure 4B and C. Red and white arrows indicate unsynapsed regions. (**A-2**, **B-2** and **C-2**) Computer-derived 'synthetic zygotene bivalents' composed of left axial element and their mirror images. (**A-3**, **B-3** and **C-3**) Computer-derived 'synthetic zygotene bivalents' composed of right axial element and their mirror images.

Discussion

In this work, the distribution of hRad51 protein in human spermatocytes was determined. The results provide evidence for a role for Rad51 in the interactions that occur between homologues during meiotic recombination in the process of chromosome synapsis. Early zygotene cells were found to contain numerous hRad51 foci which co-localized with newly formed terminal axial/lateral element material. Most importantly, mid-to-late zygotene nuclei contained greater numbers of hRad51 foci, and these were

arranged in linear arrays, with the brightest foci usually found in regions of synapsis. By pachytene, the distribution of hRad51 altered such that the labelling of synapsed autosomes diminished to a very low level, and the asynapsed regions of the XY (with the exception of the region corresponding to Y heterochromatin) became intensely labelled. At this stage, we also observed regions of asynapsed autosomes which were intensely labelled. Late pachytene cells exhibited a further reduction in the level of autosomal hRad51 labelling, with the XY bivalent either remaining labelled by few large foci or showing no labelling at all.

The distribution of Rad51 in mouse spermatocytes differed in two respects from that found in human spermatocytes. Firstly, Rad51 was detected prior to zygotene, in the leptotene stage of prophase I. Secondly, there was a heavy accumulation of Rad51 on unsynapsed axial elements, which was not dependent upon imminent synapsis.

The distribution of hRad51 in both human and mouse spermatocytes is remarkably consistent with the distribution of Rad51 in yeast (Bishop, 1994), and indicates that Rad51 functions in meiotic recombination as a component of early recombination nodules. Apparently the function of Rad51 has been conserved from *S.cerevisiae* to *Homo sapiens*.

Premeiotic Rad51 distribution

We have found no evidence to support the earlier observation that in mouse spermatocytes there is an accumulation of Rad51 as early as premeiotic S phase (Plug *et al*., 1996). Indeed, the earliest stage at which we were able to detect Rad51 was at zygotene in man, and leptotene in mouse. Our inability to detect Rad51 in premeiotic material is unlikely to be due to technical problems, such as insufficient antibody binding, since many hundreds of interphase nuclei (interpreted to be premeiotic/diploid), showing well defined centromere fluorescence, were screened from each testicular specimen. In addition, vivid Rad51 labelling was seen in meiotic prophase cells at the zygotene and pachytene stages. Indeed, we found that Rad51 always co-localized, both temporally and spatially, with axial/lateral element material. Plug and co-workers (1996) concluded that Rad51 plays a role prior to synapsis in a homology search, a stage that occurs prior to the formation of DSBs. However, our data indicate that any homology search occurring prior to synapsis does not involve Rad51, and is consistent with yeast data indicating that no known RecA homologues are involved in homologue pairing (Weiner and Kleckner, 1994).

Rad51 distribution during zygotene

Zygotene was the earliest stage at which Rad51 foci were localized in humans. Foci always co-localized with axial/lateral element proteins, indicating that the development of discernible Rad51 foci occurs subsequently to, or simultaneously with, axial element development. During zygotene, we observed a strong correlation between hRad51 foci at sites of synaptic initiation and early recombination nodules (e.g. compare the focus marked with a red arrow in Figure 1A with 'zygotene nodules' in Albini and Jones, 1987). The increase in the number of Rad51 foci between early and late zygotene (Table I) parallels the reported increase in the number of (early) recombination nodules, correlated with the increase in the amount of synaptonemal complexes present (Rasmussen and Holm, 1978). The discrepancy between the numbers of early recombination nodules (mean = 101 per late zygotene nucleus; Rasmussen and Holm, 1978) and the numbers of Rad51 foci (means of 110.2 and 152.1 for early zygotene and zygotene respectively) may be accounted for by the difficulty in identifying early recombination nodules in serially sectioned nuclei (Stack and Anderson, 1986). The results are therefore consistent with previous studies in which Rad51 was proposed to be a component of early recombination nodules in mouse, chicken and lily (Ashley *et al*., 1995; Terasawa *et al*., 1995; Ikeya *et al*., 1996; Anderson *et al*., 1997).

Our observation of the asymmetric distribution of Rad51 on zygotene chromosomes of both mouse and human spermatocytes that appear to be about to synapse suggests that synapsis occurs between regions of chromosomes that have associated Rad51 foci and homologous chromosomal regions that are free of Rad51. This observation is in agreement with the immunofluorescence data reported for lily (Terasawa *et al*., 1995) and recent immunoelectron microscopy data for rat and mouse (Moens *et al*., 1997), but is at odds with the immunofluorescence data for mouse, indicating that synapsis occurs as a result of fusion of corresponding Rad51 foci (Ashley *et al*., 1995). The data reported here and elsewhere (Terasawa *et al*., 1995; Moens *et al*., 1997) is supported by *in vitro* biochemical data showing that the pairing of homologous DNA molecules occurs between a hRad51 nucleoprotein filament and naked homologous DNA (Baumann *et al*., 1996). Indeed, extrapolation from the biochemical data suggests that not only is there no requirement for fusion of Rad51 foci on synapsing homologues, but that such a distribution would be inhibitory to homologous pairing and DNA strand exchange.

Rad51 distribution during pachytene

The occurrence of a few persistent, but faint, Rad51 foci into pachytene in mice and their numerical distribution in chickens led to the conclusion that Rad51 may be a component of late recombination nodules (Ashley *et al*., 1995). Persistent Rad51 foci were also observed in human spermatocytes, but their distribution was unpredictable, occurring on some but not all bivalents and sometimes occurring in small clusters (Figure 2C, green arrow). We conclude from this and earlier data (Ashley *et al*., 1995) that there is a poor correlation between Rad51 foci and late recombination nodules. Indeed, an average of 30.4 pachytene foci were seen, compared with an average of 46.2 nodules observed in microspread human spermatocytes (Solari, 1980). At least one late recombination nodule must exist per bivalent to ensure proper chiasmatic disjunction, and yet many bivalents were observed that were totally free of anti-Rad51 labelling. Additionally, we found that some of the foci observed during pachytene were clustered, ruling out an absolute link with late recombination nodules/chiasma formation (Hultén, 1974; Solari, 1980).

Changes in the distribution of hRad51 from regions of synapsis to asynapsis

In the studies of human spermatocytes, we observed a change in the distribution of hRad51 from regions that are

actively undergoing or have recently undergone synapsis at zygotene, to the XY and asynapsed regions of autosomal bivalents at pachytene. The labelling of the XY persists in human spermatocytes until late pachytene. This change in the distribution of Rad51 from regions where it may be promoting DNA strand exchange between homologous chromosomes, to regions where it has no such role, indicates that as prophase progresses hRad51 may have a secondary, as yet unknown role. In mice, numerous exceptionally large Rad51 foci were found along the unpaired axis of the X chromosome in early pachytene. By the time the XY began to desynapse these foci had disappeared (Ashley *et al*., 1995). This distribution is slightly different from that seen in humans, as both the X and Y are intensely labelled in humans, with the exception of the region of the Y axial element that corresponds to its large heterochromatic block (Figure 2B). The failure of the Y heterochromatic block to associate with the hRad51 recombinase is of interest given the fact that heterochromatin is known not to participate in crossing over (Hultén, 1974; Stack, 1984). Whether autosomal heterochromatin is also free of hRad51 is presently unknown.

Electron microscopic studies of microspread human meiocytes indicate that asynapsed axial elements exhibit a characteristic thickened appearance accompanied by the presence of excrescences (Saadallah and Hultén, 1986). Our observation of intense hRad51 labelling in the exact regions where thickening and excrescences are observed leads us to suggest that Rad51 accumulation and axial thickening/excrescence accumulation are the same process. This process may involve the accumulation of proteins other than Rad51, and it is of interest to note the recent observation of the co-localization of hRad51 with Brca1 (Scully *et al*., 1997).

In conclusion, we find that the human Rad51 protein is a component of early, but not late, recombination nodules, indicating that it plays a role in the interhomologue interactions that occur during meiotic recombination, which are intimately associated with synapsis during meiotic prophase I. The results provide *in vivo* support for biochemical studies in which Rad51 was shown to catalyse homologous pairing and DNA strand exchange *in vitro*.

Materials and methods

Testicular specimens

Human testicular material was obtained from two 45-year-old males of proven fertility. Male 1 had an open incisional biopsy (size ~2 mm^3) in association with a reverse vasectomy operation under general anaesthesia. A smaller amount of testicular tissue (3–4 tubules of ~2–3 mm length) was obtained from Male 2 via needle biopsy under local anaesthesia. Mouse testes were obtained after cervical dislocation from one sexually mature male Balb C mouse, from stocks maintained at the University of Birmingham.

Microspreading

Testicular tissue was macerated in Earls balanced salt solution (EBSS) to produce a thin cell suspension, and microspread according to the method described (Barlow and Hultén, 1996), with slight modifications. One drop of cell suspension was mixed with one drop of 0.003% Lipsol solution on a clean microscope slide and allowed to stand for 10 min. Six drops of 2% ultrapure formaldehyde (TAAB) containing 0.02% SDS pH 8.4 (sodium borate) were added for 10 min, after which the slides were dipped briefly in distilled water and air dried. Slides were then blocked in PBT [phosphate-buffered saline (PBS), 0.15% bovine serum albumin (BSA), 0.1% Tween-20] for ~30 min.

Immunocytogenetics

Antisera against hRad51 were raised in two rabbits using homogeneous protein purified from *Escherichia coli* (Benson *et al*., 1994). The anti-hRad51 serum (FBE-1 or FBE-2) or pre-immune sera were used at a dilution of 1:1000 in combination with serum GS (1:5000) in PBT overnight at room temperature. Serum GS was obtained from a patient with scleroderma and has been shown to label centromeres (Earnshaw and Rothfield, 1985). Slides were then given three 5 min washes in PBT before a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Sigma), combined with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-human IgG antibody (Sigma) were applied, each at a concentration of 1:500 in PBT for 4 h at room temperature. After three 5 min washes in either PBT or 4× SSC, 0.05% Tween-20, followed by a brief rinse in distilled water, the slides were air dried and stored at –70°C.

Upon removal from the freezer, slides were stained in 4′,6′-diamidino-2-phenylindole (DAPI; 0.2 μg/ml) in 2× SSC, before a brief rinse in water. Vectorshield (Vector laboratories) was applied immediately beneath a coverslip. Slides were viewed with a Zeiss axioskop epifluorescence microscope fitted with a Pinkel number 1 filter set and a cooled charge-coupled device (Photometrics). Images were captured on a Power Macintosh 8100/80 with Smartcapture software (Digital Scientific). Images of cells in which labelling with anti-Rad51 antibodies was observed were captured and a record made of the cell's position using an England finder (Agar Scientific). Slides were then washed in three changes of either PBT or 4× SSC, 0.05% Tween-20. Serum A1 (knuf) was applied at a concentration of 1:1000 in PBT for periods ranging from 2 h to overnight. Serum A1 (knuf) was raised against rat SC in rabbits and predominantly recognizes SCP3, a lateral element component (Lammers *et al*., 1994). After three washes in either PBT or 4× SSC, 0.05% Tween-20, a secondary FITC-conjugated goat anti-rabbit antibody (Sigma) was applied at a concentration of 1:500 in PBT for 2–4 h. All antibody incubations were conducted at room temperature in a moist chamber. Slides were re-counterstained in DAPI and observed as described above. Cells previously imaged were re-examined using the England finder references. For publication purposes, image contrast was enhanced and the green component of images pseudocoloured white for enhanced clarity. DAPI counterstain is not shown.

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